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Cyclosporine induces different responses in human epithelial, endothelial and fibroblast cell cultures

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Cyclosporine induces different responses in human epithelial, endothelial and fibroblast cell cultures.

Background. Nephrotoxicity, accelerated atherosclerosis, and graft vascular disease are common complications of cyclosporine long-term treatment characterized by a wide disruption of organ architecture with increased interstitial areas and accumulation of extracellular matrix (ECM). How cyclosporine induces these changes is not clear, but it is conceivable that they are the sum of changes induced at the cell level.

Methods. We studied the effects of cyclosporine on human endothelial (HEC), epithelial (HK-2), and fibroblast (MRC5) cells. Cell proliferation was evaluated by cell counting, apoptosis and collagen production by enzyme-linked immunosorbent assay, and nitric oxide by measuring the concentration of nitrite/nitrate in the cell supernatant. (α1)I and (α2)IV collagen, matrix metalloprotease-9 (MMP9), and tissue inhibitors of metalloprotease-1 (TIMP-1) mRNA levels were measured by reverse transcription-polymerase chain reaction. Proteolytic activity was evaluated by zymography.

Results. Cyclosporine showed a marked antiproliferative and proapoptotic effect on endothelial and epithelial cells. Fibroblast growth was not affected by cyclosporine. Nitric oxide was up-regulated by cyclosporine in epithelial cells and fibroblasts but not in endothelial cells. (α1)I and (α2)IV collagen synthesis was increased in cyclosporine-treated endothelial and epithelial cells, respectively. Proteolytic activity was increased in endothelial and epithelial cells. TIMP-1 mRNA was up-regulated by cyclosporine in fibroblasts.

Conclusions. Our results demonstrate that cyclosporine exhibits an antiproliferative effect on endothelial and epithelial cells. This effect is associated with induction of apoptosis probably via nitric oxide up-regulation in epithelial cell cultures. Cyclosporine treatment induces ECM accumulation by increasing collagen synthesis in endothelial and epithelial cells and reducing its degradation by up-regulating TIMP-1 expression in fibroblasts. We conclude that cyclosporine affects cell types differently and that the disruption of organ architecture is the result of multiple effects at the cell level.

Key words: matrix turnover, apoptosis, cell proliferation, transplantation, nitric oxide.

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Long-term treatment with cyclosporine (CsA) has led to the recognition of some major and limiting side effects such as nephrotoxicity [1–3] and accelerated atherosclerosis [4]. Nephrotoxicity is mainly characterized by interstitial fibrosis and tubular atrophy [5], but glomerulosclerosis has also been described [6]. Myointimal proliferation is the hallmark of the graft vascular disease associated with CsA treatment; however, changes in the endothelial cells lining blood vessels are also prominent with increased tissue factor expression [7] and endothelin production [8]. Moreover, endothelial cell and smooth muscle cell both concur to the deposition of matrix and therefore to the pathogenesis of vascular lesions [9].

Although it has been suggested that the accumulation of extracellular matrix (ECM) may be a secondary effect of CsA-induced organ hypoperfusion [10], the mechanisms underlying CsA-induced injury are not completely clear. New evidence supports a direct effect of CsA on endothelial and parenchymal cells [11, 12]. The response of each particular tissue or organ to external stimuli depends mainly on its characteristic cells and architecture. Thus, renal vascular and tubulointerstitial changes as well as sclerosis of glomeruli and graft vascular disease may represent the effect of phenotypic alterations induced by CsA on cells of different compartments, such as tubules, interstitium, and vessel wall. The aim of our study was to evaluate the in vitro effects of CsA on human fibroblasts, human microvascular endothelial cells, and human renal epithelial cells on both cell proliferation and ECM turnover.

METHODS

Materials

Cyclosporine was obtained from Sandoz Pharmaceutical (Milan, Italy). RPMI 1640 medium, minimal Eagle's medium (MEM), fetal calf serum (FCS), trypsin, penicillin, and streptomycin were supplied by Sigma-Aldrich (Milan, Italy). Agarose and loading buffer were obtained

from FAPA Pharmaceutical (Pavia, Italy). Precast gels for zymography were from BioRad (Milan, Italy).

Antibodies

Rabbit antihuman factor VIII and rabbit antihuman cytokeratin were from Dako (Milan, Italy). Rabbit antihuman type I and IV collagen antibodies were kindly donated by Dr. Liliane Striker (University of Miami, Miami, FL, USA). TRITC-labeled phalloidin was from Sigma (Milan, Italy).

Cell and culture conditions

Human renal tubular epithelial cells (HK-2) and human skin fibroblasts (MRC-5) were obtained from American Type Culture Collection (Rockville, MD, USA) and were cultured, respectively, in keratinocyte medium enriched with keratinocyte supplement and in MEM supplemented with FCS (10%). Human microvascular cells (HECs, human endothelial cells) were obtained from human skin after collagenase (0.1%) digestion. Endothelial cells were characterized by their positivity for anti-factor VIII and antithrombomodulin antibodies and by their negativity for anticytokeratin antibodies. Also, cell morphology and the arrangement of F-actin filaments stained with phalloidin were used to differentiate endothelial cells from smooth muscle cells and fibroblasts [13]. HECs were cultured in RPMI supplemented with FCS (10%). They were passaged using trypsin-ethylenediaminetetraacetic acid (EDTA) solution and were used at passages 7 through 13.

Cell proliferation

Experiments were carried out adding CsA at a dose of 800 ng/mL or at scalar doses (50, 200, 400, and 800 ng/mL) to subconfluent cells cultured in 24-well plates. Nontreated cells were used as control. After 24 hours of incubation for the dose dependence and after variable intervals of time for the time-course experiments (24, 48, and 72 hours), cells were counted using a NeuBauer chamber. Cell viability was assessed by trypan blue exclusion.

Apoptosis

Subconfluent cells were treated with CsA (800 ng/mL) for 24 hours, washed with phosphate-buffered saline (PBS), and lysated in lysis buffer. The amount of cytoplasmic mononucleosomes and oligonucleosomes was determined by enzyme-linked immunosorbent assay (ELISA) using a cell death detection ELISA kit (Boehringer Mannheim, Mannheim, Germany). Briefly, 20 μL of cell lysate, corresponding to 1×10^3 cells, were placed into a streptavidin-coated microtiter plate. Subsequently, antihistone-biotin and peroxidase-conjugated anti-DNA antibodies were added and incubated for two hours. The antihistone antibody binds to the histone component of the nucleosomes and fixes the immunocomplex to the streptavidin-coated plates. After an extensive washing to remove unbound material, the amount of nucleosomes was quantitated by the peroxidase retained in the immunocomplex. Peroxidase was determined photometrically after adding 2,2′-Azino-di[3-ethylbenzthiazolin-sulfonat] as substrate. The apoptosis was also quantitated by flow cytometry using propidium iodide as described by Nicoletti et al [14]. Briefly, cells were washed with PBS and fixed in 70% alcohol. After a 30-minute incubation with propidium iodide (40 μL/mL), cells were analyzed by flow cytometry using a Becton Dickinson FACScan.

Nitric oxide assay

Since nitric oxide (NO) is rapidly converted to its metabolites, we measured its more stable products, nitrite/nitrate, using a two-step chromogenic assay. Briefly, subconfluent HK-2 and HECs were incubated with MEM containing CsA (800 ng/mL). After 24 hours of incubation, supernatant was collected, and cells were detached and counted. The assay was performed in 96-well plates by adding assay buffer, supernatant (100 μL), and nitrate reductase-containing solution (50 μL) to convert nitrate to nitrite. Plates were incubated two hours at room temperature, and Griess reagents (100 μL) were then added to each well to transform nitrite into a deep blue azo compound [15]. The concentration of nitrite was determined by comparison with a standard curve made in the presence of known amount of nitrite/nitrate.

Extracellular matrix turnover

To study the ECM turnover, α1(I) and α2(IV) collagen subchains, 92 kD metalloprotease (MMP9), and tissue inhibitor of metalloprotease-1 (TIMP-1) mRNA levels were measured with a method that combines in situ reverse transcriptase (RT) to polymerase chain reaction (PCR) [16]. β-Actin was used as the housekeeping gene. The PCR products were separated on a 4% agarose gel, visualized with ethidium bromide, and quantitated by an image analyzer (Fluor S; BioRad).

Zymography for the metalloproteases

Cells were grown as previously described. When subconfluent, they were treated with CsA (800 ng/mL), and after 24 hours, they were further incubated in serum-free media containing 0.1% bovine serum albumin (BSA) for 24 hours. The supernatant was collected, and the cells were counted. The medium was concentrated 10 times using a 22 kD cut-off filter (Millipore, Milan, USA). An aliquot (32 μL) was loaded onto a 10% gelatin polyacrylamide gel (BioRad). After electrophoresis, the gel was incubated in 2.5% Triton X-100 buffer for one hour at room temperature and then activated in development buffer for 24 hours at 37°C, as recommended by the manufacturer. Gelatinase activity was visualized by staining the gel with Coomassie brilliant blue. Matrix metallopro-

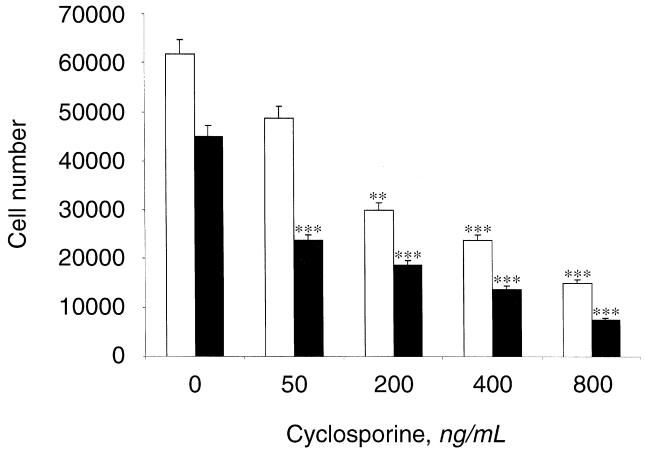

Fig. 1. Dose-dependent effect of cyclosporine on human endothelial (HEC; □) and human epithelial (HK-2; ■) cell growth. Cells were plated in a 24-well plate (5×10^4 cell/well) and allowed to attach. Fresh medium containing the indicated amounts of cyclosporine was added for 24 hours. At the end of the incubation time, cells were counted. The results are expressed as means ± SD. $^{**}P < 0.01$ vs. cyclosporine 0 ng/mL; $^{***}P < 0.001$ vs. cyclosporine 0 ng/mL.

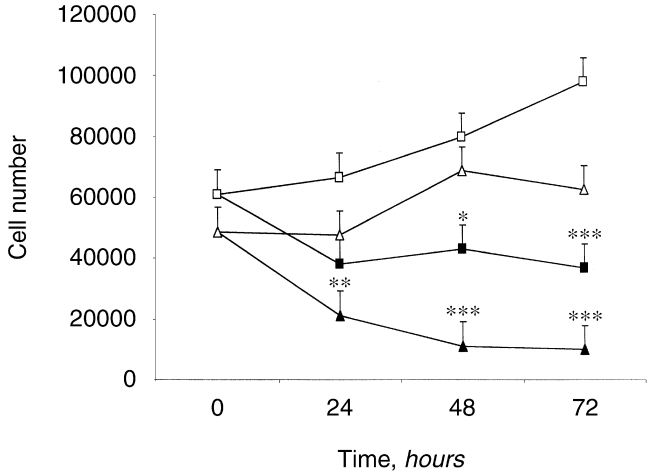

Fig. 2. Time-dependent effect of cyclosporine on HEC and HK-2 cell growth. Cells were plated in a 24-well plate (5×10^4 cells/well) and allowed to attach. Fresh medium containing cyclosporine (800 ng/mL) was added, and cells were counted at the indicated times. Untreated HEC and HK-2 were used as controls. Symbols are: (□) untreated and treated (■) HEC; (△) untreated and (▲) treated HK-2. The results are expressed as means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. untreated cells.

teases (MMP) levels were quantitated by an image analyzer.

ELISA for collagen types I and IV

Ninety-six–well plates were coated with 150 μL of conditioned medium. After an overnight incubation, wells were washed with PBS/0.05% Tween-20 and blocked for 24 hours at 4°C with PBS/2% BSA. Wells were incubated with 50 μL of rabbit antihuman type I or type IV collagen antibody (1:1000) for 1.5 hours. Wells were washed with PBS and incubated with biotin-conjugated goat antirabbit IgG antibody (1:5000 and 1:1000 for type IV and type I, respectively) in PBS/Tween-20 for one hour. Alkaline phosphatase-conjugated streptavidin (1:5000) was added for one hour followed by 1 mg/mL p-nitrophenyl phosphate for 30 minutes. Optical density was assessed at 405 nm using a Multiskan MCC/340 plate reader.

Statistical analysis

Each experiment was performed twice in duplicate or triplicate wells. Data are expressed as mean ± SEM. Two-tailed unpaired Student's *t*-test or one-way analysis of variance (ANOVA) were used to measure differences between CsA-treated and control cells. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of CsA on cell proliferation

Figure 1 shows the effect of different concentrations of CsA on HEC and HK-2 cultures. There was a decrease in cell proliferation after 24 hours of incubation with CsA when the cells were compared with untreated cells. Although HECs showed a significant reduction in cell growth at a CsA concentration of 200 ng/mL ($3.0 \times 10^4 \pm 2.9 \times 10^3$ vs. $6.1 \times 10^4 \pm 6.6 \times 10^3$ cells, CsA and control, respectively, $P < 0.01$), the effect of CsA on cell growth was more striking on HK-2 cells, which showed a decline of cell number even at a lower concentration (50 ng/mL) of CsA ($2.3 \times 10^4 \pm 3.7 \times 10^3$ vs. $4.5 \times 10^4 \pm 3.3 \times 10^3$ cells, CsA and control, respectively, $P < 0.001$). Human fibroblast cell growth was not affected by CsA at the concentration of 800 ng/mL ($5.4 \times 10^4 \pm 4 \times 10^3$ vs. $5.8 \times 10^4 \pm 0.8 \times 10^3$ cells, CsA and control, respectively, P = NS). The antiproliferative effect of CsA on epithelial and endothelial cells was also time dependent, reaching a maximum at 72 hours for the endothelial cells and at 48 hours for the epithelial cells (Fig. 2).

Pro-apoptotic effect of CsA on endothelial and epithelial cells

Next, we examined whether the antiproliferative effect of CsA occurred by the induction of apoptosis. Treatment of subconfluent endothelial and epithelial cells with CsA (800 ng/mL) for 24 hours induced a 2- and 3.5-fold increase in the percentage of apoptotic cells, respectively (Fig. 3A). CsA did not up-regulate apoptosis in fibroblast cultures (data not shown). The results obtained by ELISA were confirmed by the flow cytometry study, which showed a 20 and 30% increase of apoptotic cells,

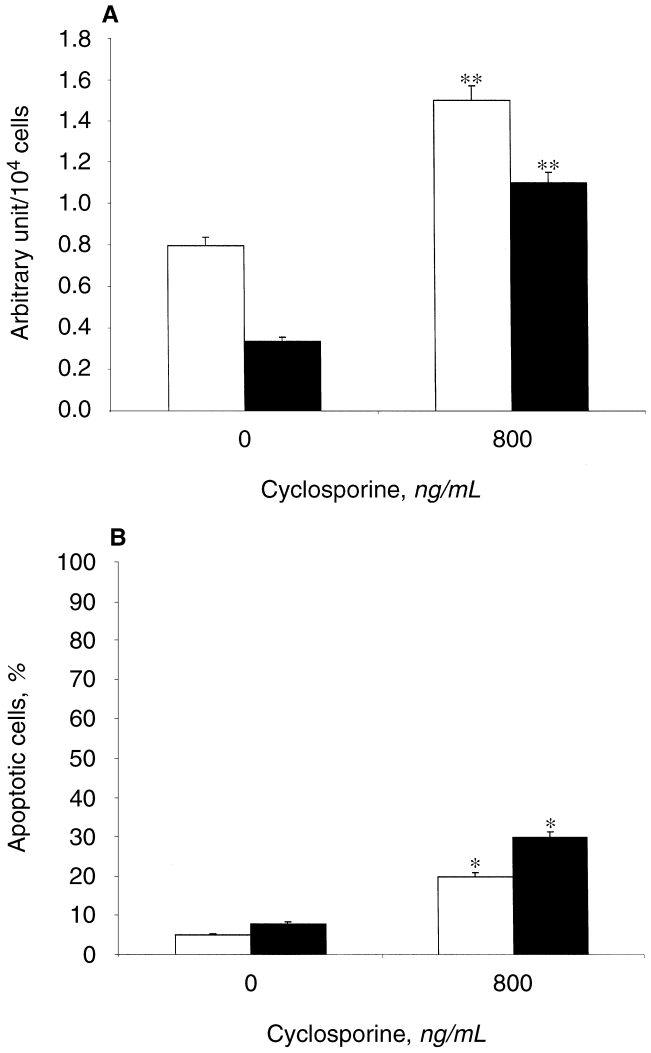

Fig. 3. Apoptosis in HEC (□) and HK-2 (■) cultures treated with cyclosporine. (*A*) Subconfluent HEC and HK-2 were incubated with cyclosporine (800 ng/mL) for 24 hours; 10^4 cells were lysated and centrifuged. Twenty microliters of supernatant were assayed for the presence of oligonucleosomal DNA using a cell death detection ELISA kit. The results are expressed as arbitrary units per 10^3 cells. $^{**}P < 0.01$ vs. untreated cells. (*B*) Subconfluent HEC and HK-2 were incubated with cyclosporine (800 ng/mL) for 24 hours. Cells were washed, fixed in 70% alcohol, incubated with propidium iodide for 30 minutes, and analyzed by flow cytometry. Results are expressed as the percentage of apoptotic cells. $^{**}P < 0.05$ vs. untreated cells.

respectively, in endothelial and epithelial cultures treated with CsA (Fig. 3B).

Effect of CsA on NO synthesis

Nitric oxide is an important intercellular messenger in a variety of pathological conditions. Since NO exhibits a well-defined antiproliferative effect [17] and its enhanced expression has been correlated with the induction of apoptosis [18], we measured its concentration in the supernatant of cells treated with CsA. The nitrite concentration was threefold increased in epithelial cells and

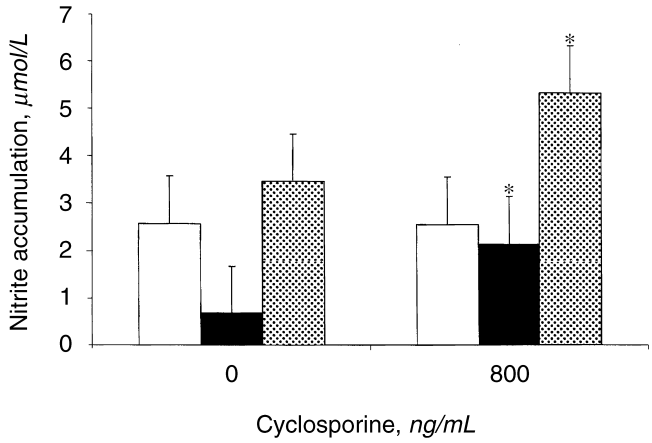

Fig. 4. Nitrite accumulation in the supernatants of fibroblasts (▩), HEC (□), and HK-2 (■) cell cultures treated with cyclosporine. Subconfluent cells were incubated with cyclosporine (800 ng/mL) for 24 hours. Media were harvested and assayed for nitrite concentration after normalizing for cell number. $^{*}P < 0.05$ vs. untreated cells.

1.5-fold in fibroblasts after CsA treatment. No difference was observed in endothelial cells treated with CsA (Fig. 4).

Modulation of extracellular matrix turnover by CsA

The effect of CsA on ECM turnover was then examined. CsA treatment did not modify the (α2)IV collagen mRNA level in endothelial and epithelial cells (Fig. 5). Although treated and untreated fibroblast cultures did not express (α2)IV collagen mRNA, CsA treatment slightly up-regulated fibroblasts (α1)I collagen mRNA (data not shown). After CsA treatment, the release of collagen type IV in the cell supernatant was significantly increased in HK-2 (7.69 ± 1.5 vs. 12.1 ± 0.45 ng/mL/10^5 cells, $P < 0.05$) but not in the fibroblast and HEC cultures (Fig. 6). Collagen type I release was significantly increased in endothelial cells (2.23 ± 0.8 vs. 3.89 ± 0.6 ng/mL/10^5 cells, $P < 0.01$), decreased in epithelial cells (5.43 ± 0.6 vs. 3.09 ± 0.4 ng/mL/10^5 cells, $P < 0.02$) and not modified in fibroblasts (Fig. 7). These results led us to consider the effect of CsA on ECM degradation. The MMP9 mRNA level was not modified by CsA in endothelial and epithelial cells (Fig. 5). MMP9 mRNA could not be detected in treated and untreated fibroblasts. CsA treatment induced an increase of collagenolytic activity in endothelial and HK-2 cells (18 and 100%, respectively, of baseline values; Fig. 8). Endothelial and epithelial cells demonstrated a different pattern of collagenolytic activity, with HEC expressing only MMP2 and HK-2 expressing both MMP2 and MMP9 (Fig. 9). Fibroblast collagenase activity was not modified by CsA. Finally, there was a marked up-regulation of TIMP-1 mRNA level in fibroblasts and HK-2 treated with CsA but not in HECs (Fig. 5).

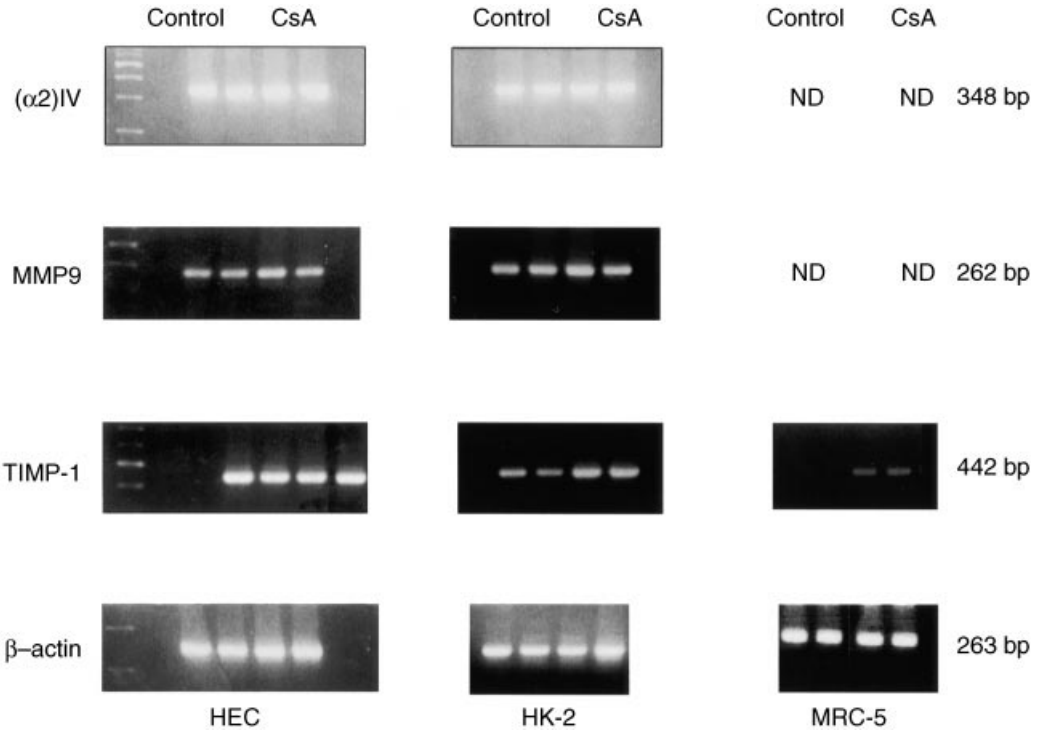

Fig. 5. Effect of cyclosporine on the expression of α2(IV) collagen, MMP9, TIMP-1 and β-actin mRNA levels in HEC, HK-2, and fibroblasts. Cells were treated with cyclosporine (800 ng/mL) for 24 hours, and the mRNA levels were analyzed by RT-PCR, as described in the **Methods** section.

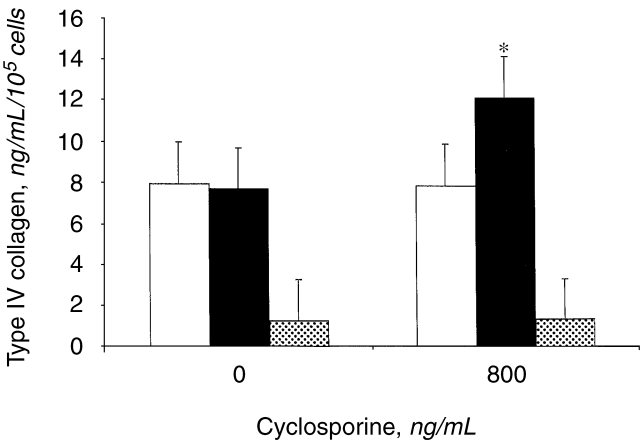

Fig. 6. Effect of cyclosporine on type IV collagen release in fibroblast (■), HEC (□), and HK-2 (■) cell cultures. Subconfluent cells were incubated with cyclosporine (800 ng/mL) for 24 hours. Media were then changed with media containing 0.1% FCS and cyclosporine for 24 hours. Cells were counted, and media were harvested and assayed for type IV collagen after normalizing for cell number. $*P < 0.05$.

DISCUSSION

Cyclosporine induces tubulointerstitial fibrosis and glomerulosclerosis [6] and accelerates vascular graft disease [4]. Although these pathological changes have been extensively described, the mechanism(s) underlying them is still undefined. We reasoned that the histopathological changes are the net effect of multiple cellular alterations induced by CsA on different cell types, which are represented in the target organs. To dissect the global effect into its individual components and to better understand the pathogenesis of CsA toxicity, we evaluated the effects of CsA on three cell types involved in CsA-induced lesions that are epithelial and endothelial cells and fibroblasts. CsA reduced cell growth in epithelial and endothelial cultures, whereas at the same concentration, it did not exhibit an antiproliferative effect on human fibroblasts. The differences in the growth curve after CsA treatment observed in epithelial cells and fibroblasts may explain the marked reduction and atrophy of tubular epithelial cells and the expansion of interstitium reported in humans and in experimental animals treated with CsA [1, 19]. Our results showed that the effect of CsA on cell growth was dependent on the length and the dosage of CsA treatment, confirming the conclusions of previous studies in vitro [12] and in humans (abstract; Esposito et al, *J Am Soc Nephrol* 8:712A, 1997) [20], demonstrating that a higher concentration and a longer treatment with CsA are associated with greater changes. Although the different modulation of cell growth induced by CsA in the three cell types used in the present study may sound surprising, different responses to CsA

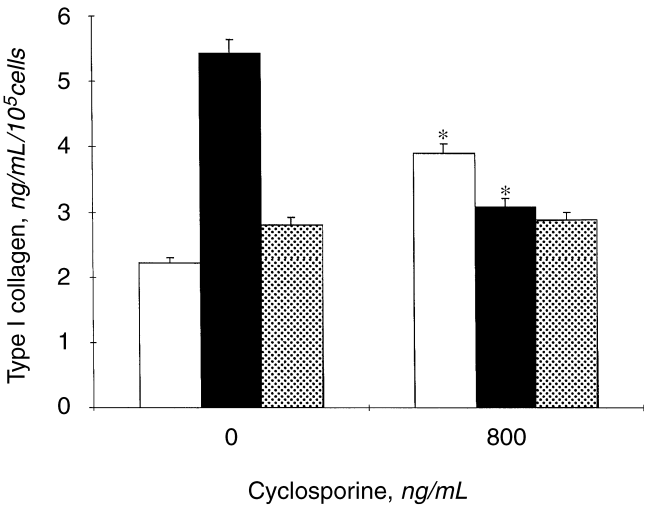

Fig. 7. Effect of cyclosporine on type I collagen release in fibroblasts (▒), HEC (□), HK-2 (■) cell cultures. Subconfluent cells were incubated with cyclosporine (800 ng/mL) for 24 hours. Media were then changed to media containing 0.1% FCS and cyclosporine for 24 hours. Cells were counted, and media were harvested and assayed for type I collagen after normalizing for cell number. $*P < 0.05$.

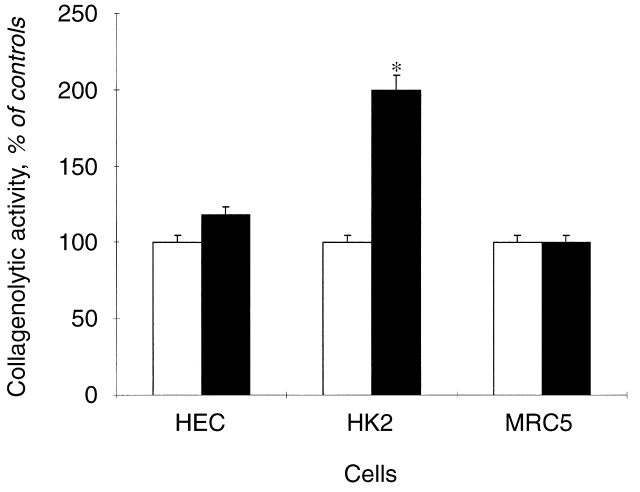

Fig. 8. Effect of cyclosporine on collagenolytic activity of fibroblasts, HEC, and HK-2 cultures. Subconfluents cells were incubated with cyclosporine (800 ng/mL) for 24 hours. Media were then changed to media containing 0.1% FCS and cyclosporine for a further 24 hours. Cells were counted, and media were diluted to normalize for cell number, concentrated with a Centricon system, loaded onto a gelatin gel and run. The bands on the zymogram were measured by densitometry. The percentage of total collagenolytic activity is shown. One hundred percent is the baseline value. Symbols are: (□) untreated cells, (■) treated cells. $*P < 0.05$ vs. control.

have been reported previously, with some reports showing an enhanced proliferation [21–23] and others an inhibitory effect on cell growth [24]. Since in our study all cell types were treated with the same concentration of CsA by the same operators, it is conceivable that the different responses to CsA depended on the cell type.

Apoptosis is one of the major mechanisms regulating cell growth. This process of programmed cell death has been indicated as the possible mechanism contributing to the loss of cell and tubulointerstitial fibrosis in CsA-induced nephrotoxicity [25]. We found that the delay in human epithelial and endothelial cell growth was associated with CsA-induced apoptosis, confirming previous data from Ortiz et al, who showed that CsA could induce apoptosis in murine tubular epithelial cells with a mechanism involving caspases 8, 9, and 3 [26]. Healy et al demonstrated that the induction of apoptosis by CsA is dose related [27]. Since we observed a more sustained effect on epithelial cells, it is possible that endothelial cells were less sensitive to CsA than epithelial cells at the concentrations we have used in the present study.

Nitric oxide has been shown to have an antiproliferative effect mediated by the induction of apoptosis [18, 28]. An up-regulation of NO synthesis paralleled the induction of apoptosis in epithelial cell cultures treated with CsA, suggesting that NO may play a role in CsA-induced apoptosis. NO synthesis was also increased in fibroblasts but not in endothelial cells. The lack of apoptosis in fibroblasts in presence of an increased production of NO confirms the differences in the cell response to extracellular mediators; however, further experiments with antagonists and agonists of NO are needed to demonstrate the relationship between NO and apoptosis. We have not investigated the mechanisms leading to the increased synthesis of NO in our cells. However, others have shown an increase in the binding of the transcription factor, activated protein-1 (AP-1), in the NO synthase promoter after treatment with CsA [29]. Our results are apparently in contrast with previous in vivo studies demonstrating a reduction of NO in rats after treatment with CsA [25, 30]. These studies suggested a role for NO down-regulation in the pathogenesis of CsA-induced hypertension and in the induction of apoptosis, which may favor CsA-associated interstitial fibrosis. In vivo studies, however, measure the net production of NO, and thus, they are not able to discriminate the contribution of each cell type, which may be up- or down-regulated by CsA. Since this study examined the effect of CsA on NO production, we cannot exclude a role of other potential mediators in CsA-induced apoptosis. For example, Ortiz et al demonstrated that CsA increased the expression of the FAS receptor in murine proximal tubular epithelial cells [26]. Furthermore, CsA has been shown to increase the mRNA expression of transforming growth factor-β, a cytokine known to act as a mediator of apoptosis [19]. Finally, the changes induced by CsA in ECM composition, observed in our study and other studies may function as a pro-apoptotic signal.

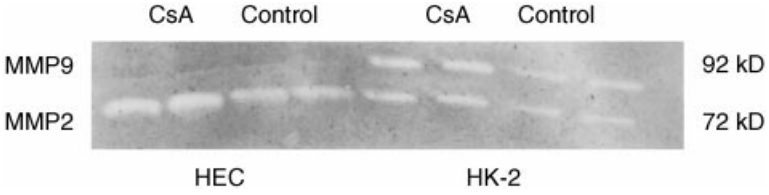

Fig. 9. Zymography of conditioned medium from HEC and HK-2 cultures treated with cyclosporine (800 ng/mL). Untreated HEC and HK-2 cells were used as the controls. The upper lane represents MMP9, and the lower lane represents MMP2. Molecular weight is shown on the right.

Extracellular matrix accumulation is the hallmark of sclerotic processes that affect vessel wall, tubulointerstitium, or glomeruli [31, 32]. Matrix turnover is a finely regulated process. In physiologic conditions, there is a dynamic balance between synthesis and degradation of ECM components. MMPs are the enzymes that degrade collagen and other matrix proteins in order to remodel basement membranes and interstitial matrix. MMP-mediated degradation is regulated by specific inhibitors called TIMPs [33]. Several cell types are involved in the processes of basement membrane remodeling and interstitial matrix turnover. However, their contribution may vary, especially in pathological conditions since cells are affected differently by environmental stimuli. In the current study, we demonstrated that CsA treatment induced a significant increase of collagen type IV in supernatants of epithelial cells but not in endothelial cells. This increase of collagen type IV synthesis was not paralleled by an increased α2(IV) collagen mRNA level, suggesting either a change in translational regulation or a down-regulation of the degradative pathway by CsA. The parallel increase of collagenolytic activity in epithelial cell supernatant was probably overcome by the significant up-regulation of TIMP-1 expression. We were not able to detect α2(IV) collagen mRNA band in treated and untreated MRC-5, even after doubling the amount of cDNA in the PCR reaction. However, the α1(I) collagen mRNA level, that is the most represented form of collagen in the interstitium, was up-regulated by CsA in fibroblasts. CsA induced a modest increase of proteolytic activity and did not change the TIMP-1 mRNA level in endothelial cells, suggesting that the degradation pathway in this cell type is only slightly affected by CsA. The marked increase of the TIMP-1 mRNA level in human fibroblasts and epithelial cells suggests that the inhibition of MMPs, rather than a decrease of their synthesis, may play a central role in the accumulation of ECM. Johnson et al have recently shown a dysregulation of ECM turnover in renal cortical fibroblasts treated with CsA [34]. They found an accumulation of collagen that was mainly due to an inhibition of MMPs in agreement with our results in skin fibroblasts. Our in vitro results confirm previous studies that show inhibition of matrix MMPs in a rat model of chronic CsA nephrotoxicity [35]. In their study, Duymelinck demonstrated an increased TIMP-1 expression in rats treated with CsA [36]. Furthermore, the cells responsible for the increased TIMP-1 message transcription were mainly interstitial cells. We restricted our study to the evaluation of collagen I and collagen IV, which represent two of the most common matrix proteins in fibrosis and basement membrane, and of MMP2 and MMP9, which are implicated in physiological and pathological processes related to matrix turnover. However, the network that regulates matrix remodeling is very complicated and difficult to unravel in one study. Based on our results, we can only speculate that CsA may also modulate in profibrotic manner other matrix proteins or collagenases not investigated in the present study.

In conclusion, CsA affects endothelial cell, epithelial cell, and fibroblast inducing changes in cell proliferation, NO production, and ECM turnover, with every cell type showing a different response. The understanding of these multiple cellular effects is important in order to elucidate the pathogenesis of CsA's side-effects and ultimately conceive future therapeutic interventions.

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